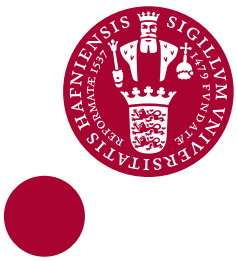

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Prospects for Applying Synthetic Biology to Toxicology: Future Opportunities and Current Limitations for the Repurposing of Cytochrome P450 Systems

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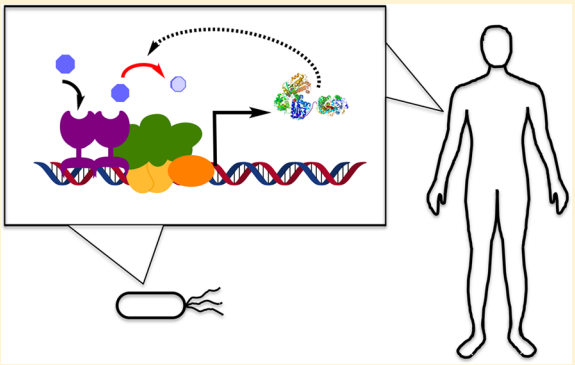
ABSTRACT: The 30 years since the inception of *Chemical Research in Toxicology*, game-changing advances in chemical and molecular biology, the fundamental disciplines underpinning molecular toxicology, have been made. While these have led to important advances in the study of mechanisms by which chemicals damage cells and systems, there has been less focus on applying these advances to prediction, detection, and mitigation of toxicity. Over the last ~15 years, synthetic biology, the repurposing of biological "parts" in systems engineered for useful ends, has been explored in other areas of the biomedical and life sciences, for such applications as detecting metabolites, drug discovery and delivery, investigating disease mechanisms, improving medical treatment, and producing useful chemicals. These examples provide models for the application of synthetic biology to toxicology, which, for the most part, has not yet benefited from such approaches. In this perspective, we review the synthetic biology approaches that have been applied to date and speculate on possible short to medium term and "blue sky" aspirations for synthetic biology, particularly in clinical and environmental toxicology. Finally, we point out key hurdles that must be overcome for the full potential of synthetic biology to be realized.



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INTRODUCTION

Nature provides a dazzling array of sensitive, flexible, and exquisitely regulated devices in the form of enzymes, receptors, regulatory systems for modulating gene expression, signal transduction pathways, and other genetically encoded "circuits". Natural evolutionary processes have generated a degree of sophistication which engineers can barely dream of emulating. Therefore, it is not surprising that efforts are now being made to "synthesize biology", i.e., to recombine existing or engineered biological "parts" (bioparts or "bio-bricks") to create complex devices for defined purposes in many areas of biology and chemistry. Toxicology provides fertile ground for the application of this emergent discipline in exploiting the biological sensors (receptors, binding proteins, enzymes, and transporters), signal transduction pathways (nuclear and other receptor-linked systems for regulating gene expression), and effector systems (enzymes and transporters) that have evolved in Nature to respond to chemical challenge. The aim of this

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perspective is to explore how synthetic biology might be used to address problems in toxicology, focusing on cytochrome P450 systems as a model for other proteins of toxicological relevance.

Synthetic biology can be defined broadly as the design, engineering, and/or recombination of biological parts, devices, and systems for useful ends (http://syntheticbiology.org/). The elements of the "devices" and genetically engineered "machines" produced by synthetic biology are usually based on and inspired by natural biological elements, but often incorporate design principles derived from engineering. Synthetic biology relies on the central importance of nucleic acids in encoding, regulating, and controlling biological systems, so while the idea of adapting Nature to anthropocentric ends is not new, it is only in the past decade that techniques for efficient, large scale DNA synthesis have allowed the facile re-engineering of biological molecules.

The redesign of biological systems can be considered at several levels of increasing complexity:[1] protein engineering; metabolic engineering; synthetic circuits and systems; the redesigning of minimal chromosomes and cells; and bio-orthogonal systems (sometimes termed "xenobiology"[2] or xenobiochemistry, terms that are somewhat ambiguous in the context of toxicology). The focus of this perspective will be on the first three of these levels, the last two being less relevant to the application of synthetic biology to toxicology. There is considerable overlap between the established field of biotechnology and the emerging discipline of synthetic biology. For the purposes of this perspective, no line will be drawn between synthetic biology and protein or metabolic engineering; while there is some debate over the precise scope and definition of synthetic biology, effectively there exists a continuum between the engineering of parts, cells, and devices, and we believe that any subdivision is arbitrary due to the dependence of more complex synthetic biology on the basic engineering of the component parts.

Mechanistic toxicology, a major focus of *Chemical Research in Toxicology*, relies on the means for reproducing, detecting, and dissecting the effects of chemicals at the molecular, cellular, and tissue levels. Indeed toxicology represents one of the first applications of synthetic biology outside pure research in the development of reporter assays for the induction of P450s by drugs and other chemicals. While not defined as synthetic biology at the time, linking the gene for a nuclear receptor in tandem with that of a reporter gene and using the natural trans-acting factors in a cell line to detect inducers combines many of the elements of synthetic biology circuits. In clinical toxicology, biosensors for the detection of drugs and other chemicals hold promise for improving both treatment with drugs and detection of poisoning. On the other hand, biologically based systems can be envisaged for the more effective treatment of overdose. Parallel opportunities can be explored in the detection of toxicants and the bioremediation of chemical spills in the environment. Finally, synthetic biology can play a supportive role in facilitating toxicological studies, e.g., in engineering of systems for biosynthesis of metabolites for structural characterization and safety assessment.

In the following sections, we explore the potential for synthetic biology to address questions in toxicology, improve the assessment of toxic responses, and mitigate the effects of toxic substances. We focus on the development of systems based on cytochromes P450 (P450s), the major metabolic system for the clearance of xenobiotics in man, as a model for the broader application of synthetic biology approaches in the field of toxicology. Four general areas emerge in which synthetic biology can be used to address toxicological problems: in the development of pharmaceuticals, in environmental toxicology, and in clinical and experimental toxicology. "Bioparts", devices and systems of increasing complexity,[1] can be envisaged in each of these areas. We aim to examine what has been done to date, focusing on key studies rather than attempting a comprehensive review of the literature but also to suggest opportunities where "blue sky" research could lead to useful advances in the next decade, and to highlight important issues that must be addressed before these can be realized.

ENGINEERED ENZYMES AND BIOCATALYTIC SYSTEMS: SYNTHETIC BIOLOGY IN THE PHARMACEUTICAL INDUSTRY

The safe development of drugs requires that the metabolism of lead compounds be characterized both qualitatively and quantitatively in terms of the metabolites produced and the enzymes responsible for clearance. The latter is critical for anticipating inhibitory drug−drug interactions where two drugs compete for the same metabolic enzyme. Additionally, testing the ability of new chemical entities to induce drug-metabolizing enzymes can indicate which compounds may accelerate the metabolism of coadministered medications. Finally, the bioactivity of metabolites may also need to be assessed depending on their quantitative importance and chemical nature. Thus, several separate issues arise: the need to identify the relevant enzymes that catalyze the clearance of a candidate drug (reaction typing), the need to assess the potential for induction, and the requirement for significant amounts of metabolites for structural identification and toxicological evaluation. P450 enzymes are center-stage in all of these issues as the principal enzymes responsible for the metabolic clearance of drugs.

Recombinant Systems for Preclinical Drug Development. Since the 1990s, recombinant enzymes have been used widely in the pharmaceutical industry for preclinical drug development studies. P450s and other proteins relevant to toxicology have been expressed in heterologous systems principally for the assessment of xenobiotic biotransformation.[3−17] Coexpression systems[18−21] including both the P450 and its redox partner(s) allowed functional reconstitution of P450 activities in the microbial host. These have been the subject of reviews and will not be detailed further here.[22−24] Likewise, the cloning of xenobiotic response elements and their cognate transcription factors enabled the development of reporter gene assays from the late 1990s, a point that will be discussed further in a later section.

These early studies provided the foundation for biocatalytic systems to address a critical bottleneck in drug development, namely, the synthesis of authentic metabolites for structural characterization and toxicological evaluation. Traditional medicinal chemistry approaches often fail to provide metabolites with the desired stereochemistry in good yield, problems that can potentially be overcome by using metabolic enzymes to modify the parental structure. Ideally, this is possible with the native enzymes responsible for the generation of the metabolites in question *in vivo*,[25,26] but in practice, bioreactors containing unmodified enzymes are limited by the total turnovers that can be achieved with unstable, promiscuous, low efficiency catalysts such as the drug-metabolizing P450s.

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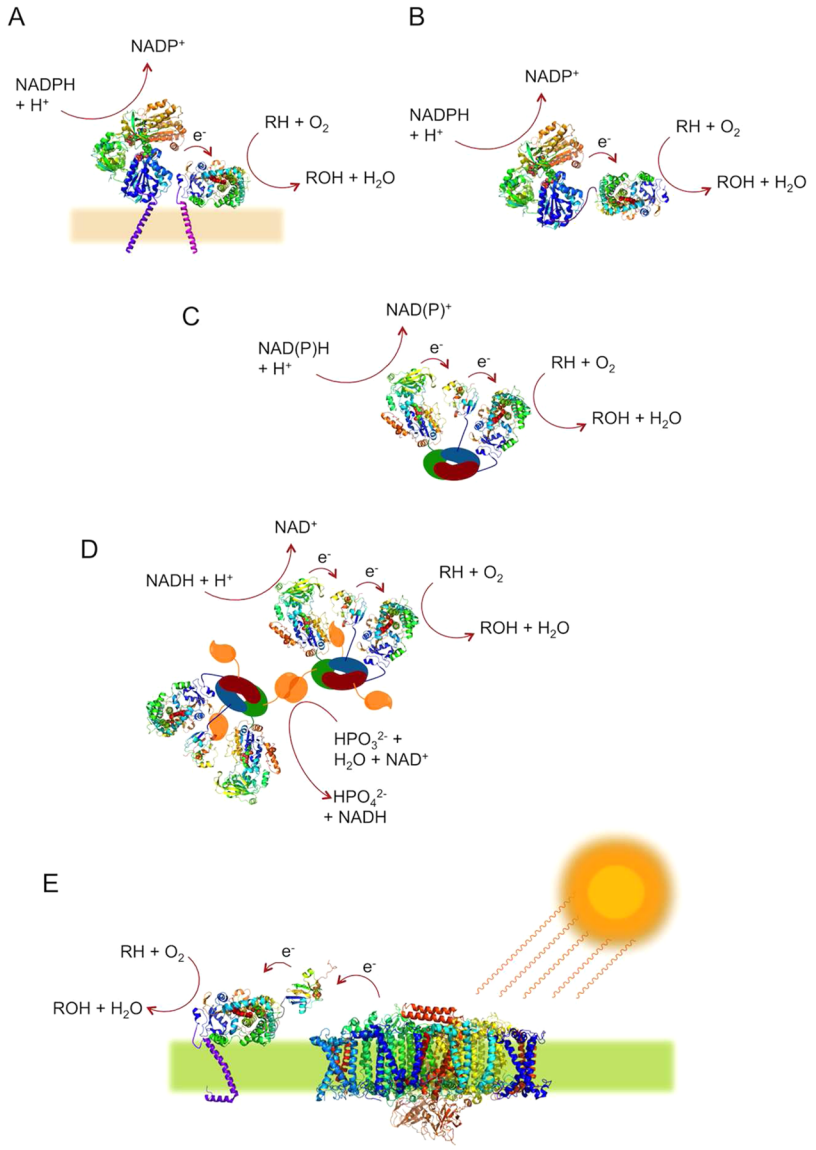

Figure 1. Alternative redox systems for supporting P450 catalysis. (A) Coexpression of P450 with its cognate redox partner in membranes; (B) fusion of the P450 with its redox partner; (C) the Puppet system, where $P450_{cam}$ and its redox partners, Pdx and PdR, are fused to the separate elements of the annular PCNA structure;[55] (D) the Puppet system modified by the fusion of phosphite dehydrogenase monomers (shown in orange), enabling cofactor regeneration and self-assembly of catalytic units into a higher-order, multimeric, hydrogel structure;[56] and (E) P450 supported by photosystem I.[70] For simplicity, the remainder of the photosynthetic electron transport chain is not shown.

Moreover, it is often useful to enhance the production of minor metabolites relative to major ones to improve their recovery.[27]

Biocatalytic Systems for Metabolite Biosynthesis. Protein engineering is a fundamental enabling technology for synthetic biology and a well-established tool for biotechnology that has been applied to improve the substrate specificity, stability, and catalytic efficiency of P450s for industrial use. Most studies have concerned $P450_{BM3}$ (CYP102A1), a "workhorse" P450 renowned for its high turnover rate on native substrates and ease of production.[28] Studies by various laboratories,[29−34] which have been reviewed extensively elsewhere,[35,36] have optimized CYP102 enzymes for use in bioreactors culminating in a set of CYP102 mutants that have been applied commercially.[30] Less attention has been paid to engineering the conventional "drug-metabolizing" P450s, but scope exists for further application of these forms due to their favorable substrate specificity, especially in regard to their stereo- and regioselectivity. Two key limitations exist to both types of biocatalysts: the stability of the enzymes, both to temperature and oxidative damage due to uncoupling during substrate turnover; and the efficient supply of electrons.[37]

The interaction of P450s with their redox partners is critical for effective catalysis but typically the most difficult aspect of systems to optimize. Coexpression and effective coupling of the P450 with its cognate NADPH:P450 reductase (CPR) are generally important for efficient reaction kinetics in heterologous expression systems,[18,19] and various means of achieving this have been developed (Figure 1). Fusion proteins, in particular, are attractive for multiple reasons[38] (Figure 1B). In theory, tethering the P450 and reductase proteins should enhance the rate of electron transfer from NADPH to the P450 since the effective local concentration of each domain is increased by the fusion. Consistent with this idea, cytochrome $P450_{BM3}$ from *Bacillus megaterium*, a naturally occurring P450-

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reductase fusion, exhibits the fastest characterized turnover of any P450 (~17,000 min^{-1} for hydroxylation of arachidonic acid) and is often cited as an example of the potential benefits of a P450-reductase fusion.[39] Mammalian P450s have been expressed as fusion proteins joined to the NADPH:P450 reductase enzyme[40−45] with mixed results. Overall, fusions have failed to yield the expected improvements in specific activity; in most cases, the rate of P450 catalysis was comparable with or decreased compared with that obtained from reconstitution or coexpression of the separate P450 and CPR domains,[44,46] and evidence has been obtained that intermolecular electron transfer occurs in addition to or in place of electron transfer between the fused domains of a single polypeptide. This has been interpreted as indicating that intramolecular electron transfer or P450 function is not well coupled in these fusion proteins, potentially due to suboptimal positioning of the NPR-P450 interaction interface or misfolding, leading to many efforts (for the most part, unpublished) to optimize the connecting linker.[47−49]

Besides the potential for enhanced reaction kinetics, expression of P450-reductase fusion proteins could simplify the functional retargeting of P450s to different organelles in eukaryotic hosts, enable the use of alternative electron donors besides soluble NADPH, and potentially simplify combinatorial cloning efforts if a reliable reductase fusion module could be developed rather than trial-and-error testing of fusions. In this regard, the LiCRED and related systems are promising[50−52] but need to be tested with a wider range of P450s.

Immobilized Enzyme Systems. Enzyme immobilization is attractive in the context of metabolite production systems. In addition to the kinetic advantages of colocalizing P450s and their redox partners described above, colocalization of a high concentration of active sites can facilitate spatially efficient turnover of substrates and improve reuse and product separation[53] (concepts of enzyme immobilization are reviewed in[54]). Importantly, colocalization of different domains can allow optimization of their relative position to enhance electron transfer, as exemplified in new biological scaffolding approaches. $P450_{cam}$ from *Pseudomonas putida* functions in a tripartite system receiving electrons from putidaredoxin reductase (PdR) via putidaredoxin (PdX). These three proteins were colocalized using a self-assembling system that exploits the heterotrimeric proliferating cell nuclear antigen (PCNA) from *Sulfolobus solfataricus* in a system, described as PCNA-utilized protein complex of P450 and its two electron transfer-related proteins (PUPPET)[55] (Figure 1C). The P450 and its two redox partners were each expressed as fusions with a PCNA monomer and when combined in equimolar concentrations *in vitro*, the PCNA monomers assembled as heterotrimers to colocalize the fused catalytic components. Optimization of both the relative position of the three domains and the affinity between the elements of the scaffold was necessary, but ultimately, the resulting system supported faster reaction rates (measured as NADH or oxygen consumption or product formation) than a mixture of the three individual proteins without the PCNA fusion tags.

Recently, the PUPPET system was developed further into a supramolecular scaffold where the PUPPET heterotrimers were cross-linked using fusions to homodimeric phosphite dehydrogenase[56] (Figure 1D). In this system, the PCNA monomers bear $P450_{cam}$, PdX, or PdR at the C-terminus and a phosphite dehydrogenase at the N-terminus. The self-assembling heterotrimers become cross-linked through the dimerization of phosphite dehydrogenase. Furthermore, the system provides a cheap NADH regeneration mechanism (via oxidation of phosphite) colocalized with PdR. The oligomeric protein complexes form a gel that is insoluble in water, and although it has a slower k_{cat} than the PUPPET system, the potential cofactor savings, and stability of the system (it can be recycled a limited number of times) make it attractive for the bulk generation of metabolites. The PUPPET system has also been used very recently to facilitate the immobilization of $P450_{cam}$, PdX, and PdR on a solid support.[57] Retention of the proteins on the support was promoted by engineering disulfide bonds between the PCNA monomers. The approach was successfully applied to intact $P450_{BM3}$, but separation of the enzyme into individual domains appeared to compromise electron transfer.[57]

Immobilization has also been developed *in vivo* in engineered *E. coli* cells. Previously, *E. coli* have been engineered to produce poly-3-hydroxybutyrate (PHB), a biopolymer that forms insoluble granules inside the host cell.[58,59] $P450_{BM3}$ was fused to the PHB-associated phasin protein, which localizes to the surface of intracellular PHB granules.[60] Because of the insolubility of PHB, granules decorated with functional $P450_{BM3}$ could be recovered from lysed *E. coli* via centrifugation. Solar-powered *in vitro* $P450_{BM3}$ catalysis was subsequently demonstrated by fusing a mutant $P450_{BM3}$ lacking the reductase domain to PHB granules and reducing the catalytic domain with the light-driven Eosin Y method that is described in detail in the following section.[61]

These immobilization methods potentially offer dramatic cost reductions for bulk *in vitro* catalysis and are highly accessible and relatively simple to manufacture, relying entirely on recombinant *E. coli* and self-assembly of the protein components. It remains to be seen whether these systems can also be used with membrane-associated mammalian P450s.

More Complex Systems: Bioreactors Supported by Different Redox Systems. The difficulty in reconstituting P450s with redox partners plus the expense of the cofactor have motivated the development of alternative means of supporting catalysis. The peroxide shunt can be used with P450s acting as peroxygenases.[62] However, the presence of peroxides (either supplied intentionally or generated by uncoupling using any mode of electron transfer) typically damages the enzyme and reduces total turnover. Rather, one of the most promising areas of research is in light-driven P450 reactions. $P450_{BM3}$ catalysis can be driven by a rubidium(II) photosensitizer covalently attached to the P450 domain in close proximity to the heme. These hybrid systems are functional in the absence of the reductase domain or NADPH.[63] Attachment of the prosthetic rubidium requires mutation of the enzyme and *in vitro* post-translational modification. While innovative and catalytically efficient toward native substrates, this approach is likely to have a limited breadth of application: it can only be used with *in vitro* systems and to-date has only been implemented with derivatives of $P450_{BM3}$.[64,65] It is not clear whether this approach would be successful with membrane-associated eukaryotic P450s.

A simpler and potentially cheaper and more versatile method of light-driven P450 biocatalysis uses Eosin Y, a photosensitive fluoroscein dye.[66] Photosensitized Eosin Y has been demonstrated as an effective mediator to transfer electrons to the P450 heme from a low-cost electron donor (triethanolamine). The main drawback of this system is the apparent decreased efficiency of electron transfer to the heme iron: O-deethylation of 7-ethoxycoumarin by $P450_{BM3}$ was considerably slower using

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the Eosin Y system than when supplied with NADPH. However, the Eosin Y photoreduction concept proved to be highly versatile. The system was implemented with human P450s 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4, and catalysis was demonstrated against a suite of drug and steroid probe substrates.[66] Furthermore, Eosin Y and triethanolamine are readily taken up by *E. coli*, and the method was functional when used with whole *E. coli* cells expressing P450s. While the reduced rates of catalysis make this system undesirable for enzymatic characterization, the reduction in cofactor costs makes it appealing for large scale and bulk biocatalysis applications. At the time of writing, NADPH is approximately 200,000 times more expensive on a molar basis than triethanolamine. The concentration of Eosin Y can potentially be optimized as a function of reagent cost and catalytic rate, as the dye is not consumed by the reaction, and the catalytic rate appears to increase with increasing Eosin Y concentration.

Experiments with plant P450s provide another, potentially useful paradigm. Fundamental studies demonstrated that certain P450s could be reconstituted with photosystem I in an *in vitro*, thylakoid membrane preparation, and that the P450 was reduced by ferredoxin in a light-dependent manner.[67−69] When CYP79A1 was reconstituted with photosystem I, the P450 turnover rate was almost twice that seen in reconstitutions of purified P450 and CPR. More recently, the ferredoxin was fused to the C-terminus of the P450 via a flexible linker domain, and this construct was demonstrated to be functional even in the absence of soluble free ferredoxin.[70] The authors sought to test whether further gains could be made in the efficient use of photosynthesis-derived electrons for P450-mediated biocatalysis. The fusion construct resulted in superior reaction kinetics under conditions of high ionic strength (>100 mM) as compared with the unfused system where ferredoxin was added separately but was less efficient at low ionic strengths. When expressed in tobacco plants, the fusion enzyme supported increased metabolism of tyrosine to the CYP79A1 metabolite *p*-hydroxy-phenylacetaldoxime and the glucosylated form, *p*-glucosyloxy-phenylacetaldoxime. The small size of the ferredoxin domain and the success of using a flexible linker suggest that the ferredoxin fusion module could be coupled to other P450 enzymes without affecting P450 folding or membrane association. While this work shows the potential for light-driven P450-mediated biosynthetic processes, it is yet to be seen if this system will be useful with other P450s, including those of toxicological relevance, such as forms from the CYP1−3 families.

The recent innovations in provision of reducing power to the cytochrome P450 are promising steps toward cheap, easy to implement, universal reducing systems for use in engineered whole cells and *in vitro* systems. While these are likely to have the most significant impact in the short term in the production of drug metabolites, longer term applications can be envisaged in other areas of toxicology such as bioremediation. In this context, plant-based systems may be especially relevant.

SYNTHETIC BIOLOGY IN ENVIRONMENTAL TOXICOLOGY: BIOREMEDIATION

The ability of P450 enzymes to oxidize and contribute to the degradation of persistent organic pollutants (POPs) is well-known.[71] P450 activities have been identified in the degradation of polycyclic aromatic hydrocarbons (PAHs)[72] and polychlorinated biphenyls (PCBs),[73] agricultural chemicals including organophosphates, and explosives such as trinitrotoluene (TNT)[74] and 1,3,5-trinitro-1,3,5-triazacyclohexane (otherwise known as 1,3,5-trinitrohexahydro-*s*-triazine; cyclonite; hexogen; or RDX).[75]

The scientific literature of the past 10 years has seen a continual disclosure of new P450-mediated reactions with potential bioremediation applications, including the discovery of new P450s involved in oxidation of high molecular weight PAHs[76] and the agricultural fungicide, propiconazole.[77] While the promiscuous catalytic activity of mammalian xenobiotic-metabolizing P450s often extends to environmental pollutants,[78,79] to date, more useful pollutant-degrading P450s have been identified in microorganisms isolated from contaminated environments.

The classical method of isolating new enzymatic functions from environmental samples is a powerful and unambiguous approach that highlights the potential utility of engineered P450 systems in the environment. However, it is also time-consuming and restricts the search space to organisms that can be cultured easily in the laboratory. The emergence of metagenomics has massively expedited the identification of genes associated with the degradation of environmental pollutants. For example, P450 sequences and other genes associated with alkane degradation were identified across several genera in metagenomic studies of diesel-contaminated sites.[80,81] Through metagenomic approaches, candidate enzymes from thousands of organisms can be identified without the need for microbial culture and isolation. With continued reductions in the cost of gene synthesis, it is becoming more straightforward to synthesize multiple candidate sequences for testing in laboratory organisms like *E. coli*.

While it is clear that environmental contamination itself can select for pollutant-degrading organisms that harbor useful and interesting P450s, naturally occurring bioremediation does not necessarily occur on an acceptable time scale for clean up of pollutants. Some plants exhibit a degree of tolerance toward POPs and an ability to remove them from the soil, thereby providing a potential alternative to natural microbial bioremediation, but many plant tissues accumulate rather than degrade the compounds of concern. For example, wild blackberry at a contaminated industrial site was observed to accumulate high molecular weight PAHs,[82] and some plant species extract RDX from contaminated soil but have a limited ability to metabolize the compound.[83] Phytoextraction of POPs without catabolism risks accumulation in the food chain (through consumption by herbivores) or returning the POPs to the soil if the entire plants including roots are not harvested and destroyed. Therefore, one of the most promising strategies for bioremediation of POPs is the engineering of organisms, particularly plants, with non-native catabolic enzymes.

Early efforts, particularly from the Ohkawa group, proved the feasibility of heterologous expression of P450s in plants[84−90] and phytoremediation of environmental chemicals, specifically herbicides. Subsequently, transfer of the RDX-degrading P450 to *Arabidopsis thaliana* alleviated the phytotoxic effects of RDX-contaminated soil at concentrations up to 2000 mg RDX/kg soil.[75] Coexpression of an RDX-degrading P450 with a TNT-degrading nitroreductase in *A. thaliana* resulted in resistance to both compounds at concentrations comparable with those found at contaminated sites, and accumulation of RDX and TNT in aerial tissues was decreased.[91] Several P450 enzymes have also been engineered for enhanced activity in oxidation of POPs including PAHs,[92,93] polychlorinated benzenes,[94] and monosubstituted benzenes.[95]

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BIOSENSORS

Detection of chemicals in the environment should also be amenable to synthetic biology approaches, and the engineering of biosensors to detect specific chemicals has additional potential applications in medicine and other fields. Most biosensors for monitoring chemicals in the environment or *in vivo* utilize naturally occurring microbial proteins known to bind the ligand of interest. However, in principle, engineered P450s could also be employed in biosensor devices to detect and monitor chemicals in various milieux, for both environmental and clinical toxicology applications. Xenobiotic-metabolizing P450s, by virtue of their broad substrate specificity, provide a useful starting material for the development of specialized binding proteins for specific chemicals and will be a useful addition to the battery of nucleic acid- and protein-based systems in development for chemical sensing.[96] Such engineered P450 systems could have broad applications in the detection of chemicals of many types.

The electrochemical properties of cytochrome P450 enzymes provide a means to monitor the P450 catalytic cycle electronically, and several P450 biosensor systems have been developed along these lines.[97−101] Much of the research in this area[102,103] has been motivated by the desire to deliver electrons to P450s using electrodes rather than consuming expensive cofactors during catalysis; where successful, catalysis can be achieved either (1) by the generation of hydrogen peroxide by reduction of Fe(II)-O_2 at the electrode and its subsequent use to generate the high valent, active, oxygenating species of P450 (compound I) via the peroxide shunt (which can occur in the absence of any redox partner) or (2) in a way that more closely mimics the native system, i.e., via reduction of the P450 by CPR reduced at the electrode.[103,104] It is unclear what the working lifetime of such systems would be if employed for biocatalysis, especially in light of the continual production of hydrogen peroxide at the electrode surface via electrolysis of oxygen. Therefore, it would be advantageous to engineer forms of the enzyme that are resistant to oxidative damage and also more thermostable for further development of such systems.

Arguably, given concerns over sensitivity to damage by peroxide, the application of electrochemical systems purely as biosensors may be more realistic in the short term. The ultimate goal of P450 biosensors is in point-of-care diagnostics or monitoring of environmental pollutants in the field, but devices have also been proposed for screening for drug−enzyme interactions in drug development. The majority of proposed P450-based biosensors use immobilized enzymes and measure changes in the electrochemical properties of the P450 upon interaction with a ligand. These experimental biosensors that interface enzymes with electrical circuits have been discussed in detail elsewhere, and the reader is directed to a number of excellent reviews.[105−109]

One important application of such devices is in therapeutic monitoring of drugs for which the effects are not easily detected or titrated based on response. The detection of such drugs currently relies on resource-intensive blood tests. For drugs such as immunosuppressive agents and anticonvulsants, which show a small therapeutic window and large interindividual variability in pharmacokinetics, there is a clear need for convenient, minimally invasive devices that sense drug concentrations and report them in terms of an easily detected output, similar to a blood glucose monitor. As examples, immobilized enzymes have been investigated for the measurement of the drugs, indinavir,[110,111] caffeine,[112] codeine,[113] naproxen,[114] ethambutol,[115] phenobarbital,[116] sertraline,[117] and the illicit drug cocaine.[118,119] Analogous approaches have been used for environmental monitoring, e.g., of bisphenol A,[120] 2,4-dichlorophenol,[121] pesticides,[122] and benzo[*a*]pyrene.[123]

In an extension of the basic electrochemical biosensor idea, Rusling and co-workers have developed extensive technology based on measuring genotoxicity as well as P450 activity in various electrochemical and electrochemiluminescent systems. The majority of these systems use native enzyme preparations, such as purified enzymes or liver microsomes, which are incorporated with or without DNA into thin layers of alternating charge starting with a synthetic polyion using a layer-by-layer film fabrication technique (reviewed in ref 106). Other groups have used similar approaches to measure DNA damage.[124]

To date, most studies have essentially involved coupling of more or less native (i.e., unengineered) enzyme preparations to electrodes, but there is no reason why such devices should be limited to existing enzymes. In theory, the promiscuous drug-metabolizing P450s could be evolved *in vitro* to bind with higher specificity to chemicals of interest and to generate a greater electrochemical response. Also, as with electrocatalytic systems, the limited stability of the currently available enzymes means that the shelf life and therefore the economic feasibility of such devices is determined by their half-life. Fortunately, thermostability is another property that can be improved by *in vitro* evolution.[125]

One novel approach combines the amperometric chip biosensor concept with whole cells.[126] Whole *E. coli* expressing $P450_{BM3}$ were suspended in a buffer solution containing glucose in an electrochemical cell and monitored with a potentiostat for changes in current due to the product of the oxidation of aniline upon addition of inhibitors (including alternative substrates). The system was validated by comparison with purified $P450_{BM3}$. The ability to use intact *E. coli* in this system greatly enhances the flexibility of electrochemical P450 biosensors. In addition to eliminating NADPH costs, enzyme purification becomes unnecessary, and fresh catalytic material can be prepared with minimal effort. Furthermore, it suggests the possibility of a flexible system where different P450 enzymes expressed in *E. coli* can easily be substituted in order to screen for different compounds, given an appropriate electroactive, probe substrate. The system has not yet been validated with human drug-metabolizing P450s; however, such devices may be of use in drug development to screen for inhibitors of drug metabolism by P450s or in environmental monitoring. The sensitivity may be poorer with slower enzymes, particularly in cases where expression in *E. coli* is poor. However, this concept has potential for developing compound-screening devices using other P450 enzymes.

The redox characteristics of P450s or their products provide one end point for biosensors, and the interest in electrochemical biosensors relates to the easy integration with amperometric devices developed for other biosensors, e.g, blood glucose monitors. However, other characteristics of P450s provide scope for detecting ligands in alternative ways, e.g., plasmon resonance[127,128] or oxygen consumption.[129,130] For example, the spin shift induced by the binding of a ligand to a P450 could be detected colorimetrically as a change in the absorbance of visible light in a specific wavelength range. In another, more complex example, an *E. coli*-based biosensor has been engineered that could potentially be used for the

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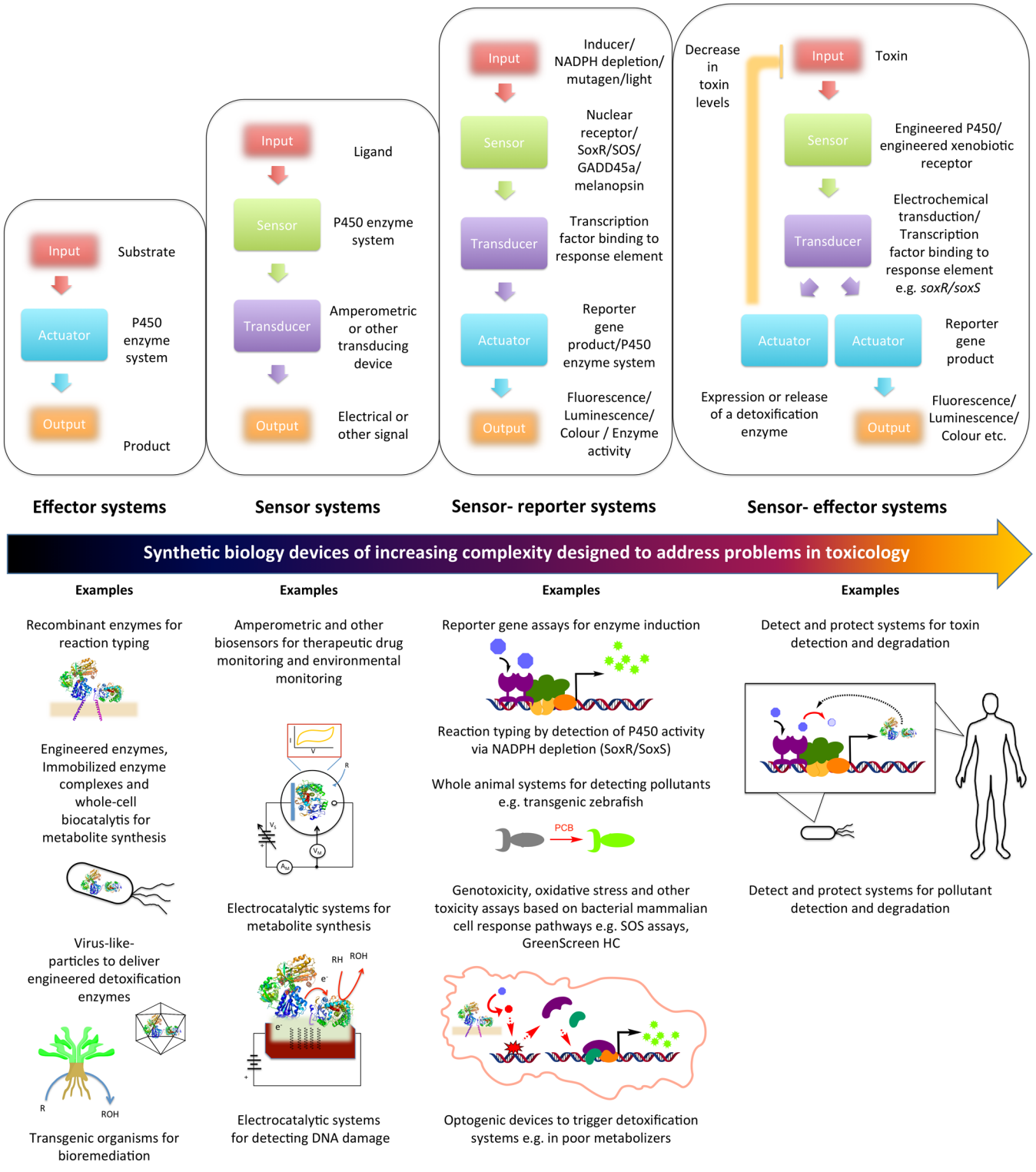

Figure 2. Levels of complexity of synthetic biology approaches that could be applied to toxicological problems with examples of possible devices. Systems can be conceptualized ranging from simple effectors comprising a catalytic complex to a sequence of interacting bioparts that enable an appropriate response to be generated to a specific stimulus. Simple sensor-type arrangements are defined here as systems where a biopart is linked to some physicochemical transducer that can allow a signal to be generated in response to a change in the biopart elicited by interaction with a specific chemical. Such systems can also be conceived of operating in reverse, as effectors, where a physicochemical (e.g., electrical) signal is used to elicit a change in the biopart to effect a specific outcome, e.g., the supply of electrons to a P450 to enable catalysis.

detection of any ligand metabolized by a P450 on the basis of NADPH consumption.[131] The *soxR* gene encodes an iron–sulfur cluster repressor protein that, when in a reduced state, represses the expression of the *soxS* gene. The soxR protein is maintained in a reduced state by the activity of native NADPH-dependent reductase enzymes, and a decrease in the availability of NADPH results in oxidation of soxR and subsequent expression of *SoxS*. By placing a fluorescent protein under the

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control of the *soxS* promoter, the activity of non-native NADPH-consuming enzymes, such as P450s, can be detected upon addition of their corresponding substrates due to a sudden decrease in the available NADPH pool. This type of system could be used with engineered P450s specific for ligands of interest. However, it would be important to ensure that this type of biosensor was both highly specialized and well coupled, as many ligands are known to trigger enhanced NADPH oxidation by promiscuous P450s, and this is not necessarily coupled to substrate oxidation.

GENETICALLY ENCODED RESPONSE CIRCUITS: AUGMENTED TOXICOLOGICAL SCREENING SYSTEMS

Genetic response circuits, such as the one in the *soxS* example above, illustrate the next step up in complexity of synthetic biology systems (as depicted in Figure 2), and there are a number of existing examples of simple response circuits used for screening for toxic effects of chemicals. These can be understood in terms of a simple paradigm linking some input (e.g., the presence of a particular chemical), through a sensor and transducer, to an output (e.g., expression of a reporter protein).[132] For many years, recombinant reporter gene assays have been used to predict the potential of new chemical entities to induce P450 expression since this can alter the metabolism of coadministered drugs causing adverse drug interactions. These assays typically link the upstream regulatory sequences for a gene of interest (usually a P450) to some easily detected reporter protein, such as a fluorescent protein. Ligands are screened in an appropriate host cell for the ability to alter the binding of nuclear receptors/transcription factors to regulatory elements, affecting the expression of the reporter gene. As well as in drug development, this concept has been applied to environmental monitoring. The presence of polycyclic aromatic hydrocarbons and related pollutants in aquatic environments can be assessed by the induction of P450s in fish. Thus, a transgenic zebrafish has been developed, in which GFP expression is regulated by the CYP1A1 promotor, allowing detection of polychlorinated biphenyls.[133]

Bioactivation of chemicals by P450s and other enzymes has also been studied using genetic response circuits. The most notable example is the SOS response which results in widespread transcriptional activation in response to DNA damage. Several systems have been developed where reporter genes are activated by the SOS response. Indeed, shortly after the development of human P450 expression in *E. coli*, P450 metabolites from procarcinogen assays were screened for activation of the SOS response[134] in a *Salmonella typhimurium* strain engineered so that DNA damage triggered the expression of β-galactosidase.[135] Subsequently, human P450s 1A1, 1A2, 1B1, 2C9, 2D6, 2E1, and 3A4 were each expressed in *S. typhimurium* along with the human CPR, enabling catalysis and response activation within a single system.[136,137] The SOS-β-galactosidase system has also been optimized in *E. coli*.[138]

Although β-galactosidase is easily assayed and can be used in very high throughput with qualitative blue/white screening on agar plates, its applications are limited compared with other systems designed for quantitative high throughput screening. *E. coli* have been engineered to produce luciferase[139] and green fluorescent protein[140−142] in response to direct SOS activation by mutagens, but to our knowledge, these systems have not been coupled to enzymatic bioactivation systems (e.g., recombinant P450s) to date.

More recently, analogous systems have been developed in yeast[143] and mammalian cells[144−154] that should more accurately reflect toxicological responses in humans. In one of the key examples, the promoter and regulatory sequences of a growth arrest and DNA damage gene, GADD45a, the expression of which is upregulated in human cells in response to DNA damage, were coupled to the expression of GFP in a human lymphoblastoid TK6 cell line.[144] This "GreenScreen HC" test is sensitive to many types of genotoxins, including aneugens which are not routinely detected in bacterial genotoxicity screens. GreenScreen HC also compares well to alternative genotoxicity tests such as the SOS-Chromotest and Mini Ames II.[155−160] This has been the forerunner of a number of similar reporter gene assays based on linking the regulatory sequences for some protein involved in a response to toxic insult (multiple mechanisms of genotoxicity, oxidative stress, etc.) to a fluorescent or luminescent reporter gene.[143,145−154] These have been reviewed recently elsewhere[161] and will not be covered in detail here. Again, however, most lack any endogenous bioactivation system to enable the detection of toxicity due to metabolites rather than parent compounds. Exceptions are tests developed in metabolically competent HepG2 cells expressing at least some phase I and II activities[146] and in primary human hepatocytes.[152]

The explosion in genotoxicity and other reporter gene screening assays in recent years based on signaling pathways for responses to toxic stimuli[161] highlights the need for an understanding of the importance of and interplay between specific key events in toxicological cascades, i.e., adverse outcome pathways[162] in order to apply the reductionist approach of synthetic biology. Notwithstanding these examples, much toxicology testing is dependent on high content screening in mammalian cell culture, and tissue- or animal-based systems.[163−166] While synthetic biology approaches would be advantageous in replacing mammalian cell-based and animal testing with less costly and more ethically acceptable alternatives, toxicity is a complex end point. Modeling of toxicological processes must include the behavior of systems at low concentrations and the existence or otherwise of threshold effects.[167] However, inspiration can be drawn from phenotypic screening systems developed for drug discovery, in which synthetic biology approaches are increasingly being incorporated (reviewed in ref 168). Conceivably, the dependence of toxicological outcomes on multiple factors can be captured by synthetic AND gates,[169] and thresholds can be introduced by careful choice of components and clever design of circuits.

SYNTHETIC BIOLOGY IN CLINICAL TOXICOLOGY: PUTTING IT ALL TOGETHER FOR HUMAN HEALTH

Numerous applications of synthetic biology can be envisaged in clinical toxicology. At one extreme, the treatment of acute toxic chemical exposure (e.g., drug overdose, poisoning, or medication error) could be augmented by delivering engineered enzymes able to detoxify the toxin. For example, engineered P450 enzymes could be delivered in virus-like-particles (VLPs),[170] which are simple, well-defined, self-assembling nanostructures that show promise for many applications.[171] The judicious choice of a VLP might enable targeting of specific tissues to enhance detoxification *in situ* of chemicals that are concentrated in or toxic to particular organs.

Analogous approaches could enable pre-emptive treatment of possible iatrogenic poisoning. Patients with genetic poly-

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morphisms in certain P450s are at risk of accumulating toxic levels of drugs when given standard doses. An alternative to avoiding the affected drugs or adjusting doses (which may be difficult for orally administered drugs or in an outpatient setting) might be the use of implanted optogenetic devices, like that developed for blood glucose control,[172] that could be triggered to express an engineered P450 as and when required. In these devices, melanopsin, which detects blue light, is expressed in a mammalian cell system. When appropriately illuminated, melanopsin triggers a signal transduction pathway leading to the expression of a target gene. In this case, the target would be an engineered P450 enzyme able to efficiently detoxify the toxin of interest. Alternatively, designer signal transduction pathways could be envisaged analogous to that developed for experimental treatment of metabolic syndrome.[173] Ideally, both of these types of system would be delivered in subcutaneous, semipermeable, encapsulated, cell implants, protected from the immune system and readily irradiated with blue light.

Moving beyond the "effector" and "sensor-reporter" (e.g., simple biosensor) paradigms to the "sensor-effector" model (Figure 2), synthetic biology devices of greater complexity can be envisaged that combine detection and treatment of toxicity, "detect and protect" systems for enhanced clearance of toxins from the body when needed. Here, the input would be the chemical of interest, and the main output would be the expression of an enzyme to degrade the toxin. This is analogous to "prosthetic networks", e.g., for controlling blood urate concentrations (reviewed in ref 174); such smart systems would couple the detection of a toxic chemical to the triggering of expression of a protective mechanism. In this case, use could be made of existing "bioparts" in the form of the nuclear receptors that sense many diverse xenobiotics and their corresponding response elements: the gene for a P450 engineered to be highly efficient at detoxification of the chemical of interest could be put under the control of this system such that its expression was induced when required. Engineering of the nuclear receptor could potentially enhance its specificity toward toxins of particular importance and limit unwanted cross-talk with other chemicals. An alternative type of switch is exemplified by Erkelenz et al., who attached the two domains of $P450_{BM3}$ to short oligonucleotides.[175] In this system, the P450 activity could be tuned by the presence of competing oligonucleotides (or potentially miRNAs). However, the need to conjugate the P450 with an oligonucleotide currently limit this approach to *in vitro* applications.

CONCLUSION AND FUTURE PERSPECTIVES

It should be clear from the discussion above that there are abundant opportunities to deploy synthetic biology approaches in toxicology (Figure 2), as in other areas of the biomedical and life sciences. Moreover, the applications outlined above for P450 enzymes could, in many cases, just as easily be envisaged for other proteins of toxicological interest. So the question arises as to why there has been relatively little uptake of synthetic biology to solve toxicological problems since its emergence as a discipline, outside of conventional protein engineering approaches for drug development.

As noted above, for synthetic biology devices using P450s to be economically feasible, P450s need to be specific for the reaction/chemical in question and, where catalysis is needed, able to sustain high turnovers with cheap sources of electrons. More effort is needed in the engineering of P450s to these ends. A key property for all applications is thermostability, as this determines the half-life of the protein at ambient as well as elevated temperatures and thereby the effective lifetime of the device or system.

One major limitation has been the lack of reliable, "drop-in" expression systems, which on average can be expected to work for most P450s, so that the diversity of forms found in Nature can be exploited. The heterologous expression of P450s remains an "art" in many respects, despite more than 20 years of continuous study. The guidelines for achieving success in expressing P450s in bacteria or yeast remain somewhat empirical; it is not possible to predict *a priori* which P450s will be straightforward to express and which problematic or which of many alternative approaches will work and why. There have been some recent successes in this regard,[176] but it remains to be proven whether these advances will be broadly applicable.

The ideal, "all-purpose" expression system would include modular, interchangeable redox partners, the levels of which could be controlled independently of the P450, and automatic coexpression of useful chaperones (e.g., genomic engineering of groES/EL to be induced concurrently with the P450).

Another important gap is the lack of a truly convenient host organism for P450-based studies. The advantage of *E. coli* is its simplicity and the absence of endogenous P450s, but functional expression is fraught by the problems outlined above. Many eukaryotes are better suited for functional expression, but analyses can be confounded by native P450s, and expression yields may be harder to detect and optimize. Moreover, mammalian cell systems are less well characterized as hosts for synthetic biology applications than *E. coli* or *Saccharomyces cerevisiae*. Other aspects to consider would include membrane composition, viability of the cells without native P450s, engineering for heme and flavin supply (i.e., a superior host cell that diverts resources toward P450 support, reducing media costs), and engineered chromosomal coexpression of beneficial chaperones. In this regard, the development of a simplified, "minimalist" cell, one of the aims of synthetic biology, could be explored. One might envisage ultimately the creation of a cell line that expresses the full battery of metabolic enzymes and transporters found in the human hepatocyte, for example, at levels that could be tuned to mimic different genotypes. Such a cell, after extensive validation, might eventually supplant hepatocytes as a test system for preclinical drug metabolism studies.

The most fertile area for synthetic biology in the short term is likely to be in biocatalysis, for the production of metabolites and other chemicals. This represents the "lowest hanging fruit", and implementing solutions from problems solved here will facilitate the approaches to other problems. Another short-term target should be the introduction of metabolic (bioactivation) systems, including but not limited to P450s, into the newer mammalian genotoxicity and other screening systems. For toxicity assays, it is important that the host cell expresses mammalian proteins, e.g., in the pathways that are activated upon perception of a toxic insult. Again, a simplified, "drop-in", engineered host cell would be useful, in which one could reasonably anticipate functional expression to be straightforward and easily quantified, and which recapitulates the required pathways for manifestation of the toxicity.

For less straightforward applications, more needs to be done to evaluate the component bioparts and how they behave. Synthetic genetic circuits are difficult to develop for several reasons:[177] precise tuning is required to optimize the level of

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expression of different elements; regulatory elements are often toxic to the host cell; cross-talk between elements of a circuit or with other cellular components can impair function of the circuit; the design rules are not well characterized for biological circuits; and the physical construction of circuits composed of many parts from different biological contexts can be problematic. For most purposes in toxicology, it may be possible to design circuits in which fewer factors need to be optimized than in conventional synthetic biology applications. This is because natural "biobricks" exist in the form of the receptors, transcription factors, and response elements that mediate the response to toxicity, and are, by definition, active in the relevant physiological or toxicological concentration range. However, again, it will be important to choose an appropriate host cell "chassis" and to tune the expression of the various components.

Advances in synthetic biology in the past decade, particularly with respect to metabolic engineering, have simplified the development of organisms that produce valuable chemicals and provided pathways for optimization. This has stimulated the transfer from lab bench to practical application. On the other hand, many of the same principles can be applied to organisms engineered for purposes such as bioremediation, but there has been minimal progress toward practical application of synthetic biology in these applications, despite the wealth of data available on suitable catabolic enzymes including engineered P450s. In order to understand the lack of progress in practical applications of synthetic biology more broadly, compared to the recent boom in synthetic biology for bioproduction, it is helpful to explore the situation concerning bioremediation, as an example in which societal and economic drivers are quite different from those for chemical synthesis.

Modern bioproduction and prospects for effective bioremediation rely heavily on genetically modified organisms (GMOs), which are regulated in most countries in order to prevent unforeseen consequences of widespread release in the environment. Consequently, bioproduction using engineered microbes that can be fully contained in an industrial fermentation has flourished while the deployment of engineered plants has been relatively slow due to the greater risk of unintended spread and cross-pollination with non-GMO species. In the case of bioremediation, most practical applications would require deployment in the field at contaminated sites, which complicates the development process in most jurisdictions.

Additionally, the market incentives for the development and deployment of bioremediation solutions are not clear. At face value, the market for bioremediation technologies would appear vast, but the picture is complicated by the varying types of compounds that require bioremediation and the distribution of sites across legal jurisdictions and between public and private ownership. For comparison, the development of organisms for the production of advanced biofuels has been accelerated by clearly defined government incentives and by what is effectively a permanent global market for the consumption of energy. No such situation exists for bioremediation. The diversity of contaminated sites, the size of each market, and the regulatory barriers to entry are unclear. It is difficult to imagine formulating a business case for private investment in developing bioremediation technologies, and it is unclear whether bioremediation research and development are capable of attracting significant government funding when it is difficult to define the scope of the problem. It might be argued that the most valuable contribution of scientists now to advance bioremediation technology is not necessarily to discover and develop new enzymes but to create and promote conversation about bioremediation with the public, government officials, and private companies.

Engagement with the wider community has been taken very seriously in the development of the synthetic biology field, e.g., through the iGEM (International Genetically Engineered Machine) competitions, in an effort to bring the public along with the tide of innovation enabled by synthetic biology, in the hope of avoiding mistakes made in the past. The fact that novel toxicological applications feature so commonly in the topics chosen by iGEM teams suggest that the scientists of the future see synthetic biology as an important means by which to address problems in the clinical, experimental, and environmental fields. While it is impossible to predict where the field will go in the next 30 years, the energy and enthusiasm with which synthetic biology is being taken up by the next generation of scientists suggests that there will be more things in heaven and Earth than can be dreamt of with our current philosophy.

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Notes

The authors declare no competing financial interest.

Biographies

James Behrendorff studied biochemistry at the University of Otago, New Zealand, before undertaking his Ph.D., studying cytochrome P450 enzymes with Elizabeth Gillam (University of Queensland, Australia). Subsequently, he has worked on projects in the areas of isoprenoid metabolic engineering (Australian Institute for Bioengineering and Nanotechnology) and industrial synthetic biology (LanzaTech, Inc., USA). James is currently researching aspects of chloroplast biochemistry at the Copenhagen Plant Science Centre (University of Copenhagen, Denmark) with a focus on biotechnological applications.

Elizabeth Gillam has followed a common thread of P450 biochemistry through most of her career, moving from drug metabolism and molecular toxicology through to pharmaceutical biotechnology, enzyme evolution, and synthetic biology. After studying Biochemistry at the University of Queensland (Brisbane, Australia), she completed her D. Phil. in the Pharmacology Department at Oxford University (UK), then spent two years as a postdoc with Professor F. P. Guengerich at Vanderbilt University (Nashville, TN, USA), before taking up an independent academic position in Pharmacology at the University of Queensland. Elizabeth returned to her core discipline in 2009, joining the School of Chemistry and Molecular Biosciences as a Professor of Biochemistry, where she leads the Biocatalysis and Biotransformations group and the Structural Biology and Biochemistry theme.

ABBREVIATIONS

CPR, NADPH:P450 reductase; CYP, cytochrome P450; P450, heme thiolate protein P450; GMOs, genetically modified organisms; iGEM, International Genetically Engineered Machine; PAHs, polycyclic aromatic hydrocarbons; PCBs,

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polychlorinated biphenyls; PCNA, proliferating cell nuclear antigen; PdX, putidaredoxin; PdR, putidaredoxin reductase; PHB, poly-3-hydroxybutyrate; POPs, persistent organic pollutants; PUPPET, PCNA-utilized protein complex of P450 and its two electron transfer-related proteins; RDX, 1,3,5-trinitro-1,3,5-; TNT, trinitrotoluene; VLP, virus-like particle

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